SEMEN ANALYSIS:

What? When? How? Why?

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BACKGROUND

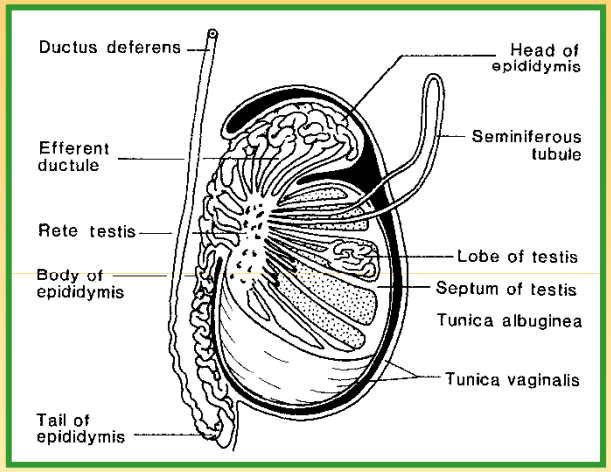
What is semen, exactly?

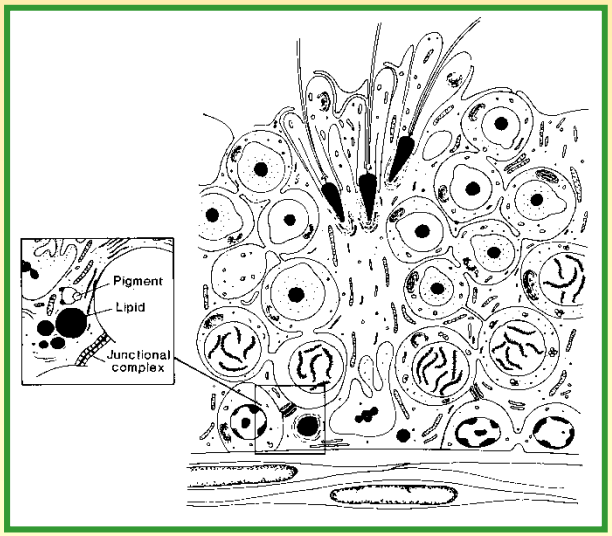
A mixture of seminal plasma and cells

- **Seminal plasma contains:**
  - ***Prostatic fluid (~30% of the volume)***
  - ***Epididymal plasma (~5% of the volume)***
  - ***Seminal vesicle fluid (the remainder of the ejaculate)***
- **The cells are:**
  - ***Spermatozoa***
  - ***Germ line cells***
  - ***Leukocytes of various types***
  - ***Bacteria***
  - ***Epithelial cells***
  - ***Occasional red cells***

BACKGROUND

Formation of the sperm cell (1)

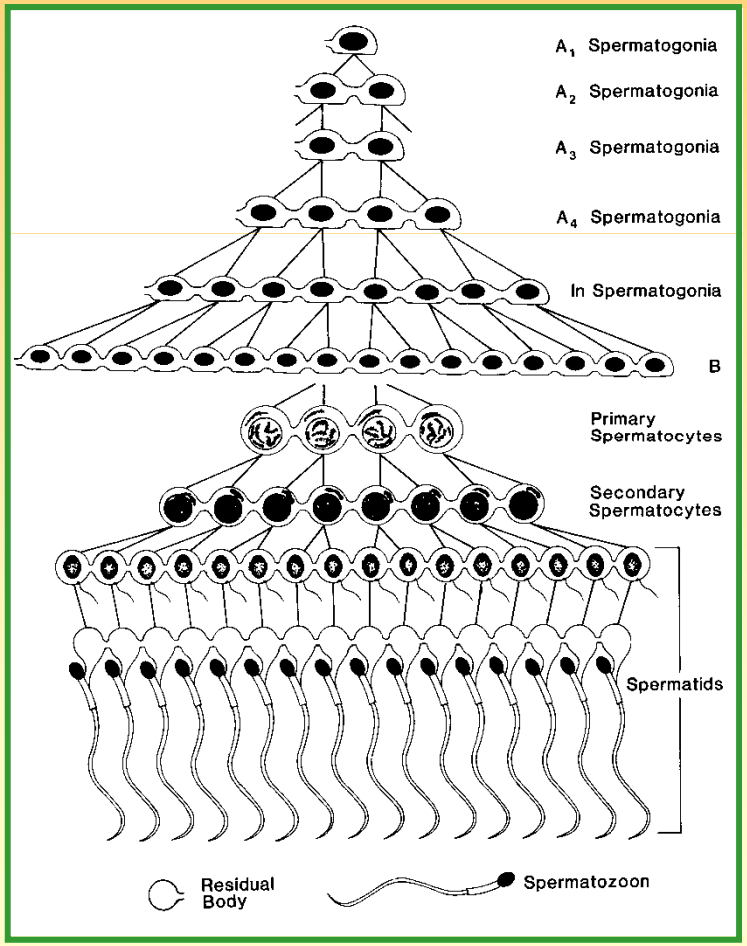




- **Formed in the seminiferous tubules, develop in close association with the Sertoli cells**
- **Start as spermatogonia (self-renewing stem cell of the male germ cell line) – located on the basement membrane**
- **The transformation from the round germ cell to the sperm cell occurs during passage to the centre of the seminiferous tubule**

BACKGROUND

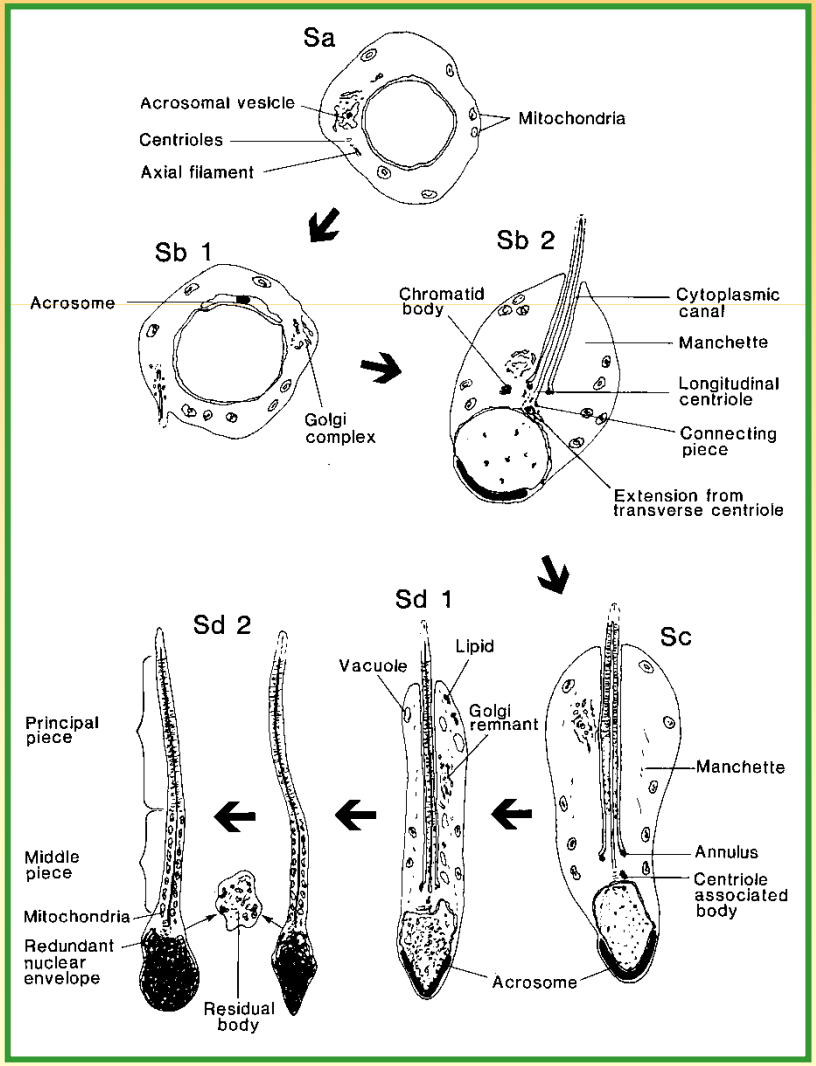
Formation of the sperm cell (2)



- **Spermatogenesis is a cascade of cell divisions:**
  - **Mitosis: spermatogonia to primary spermatocytes**
  - **First meiotic division: secondary spermatocytes**
  - **Second meiotic division: haploid spermatids**
- **This process takes 70 ± 4 days in the human – so errors will take about 3 months to show up**

BACKGROUND

Formation of the sperm cell (3)



- ***Spermiogenesis*: differentiation of the round spermatid into a spermatozoon**

- **This is the process in which sperm morphology is largely determined**

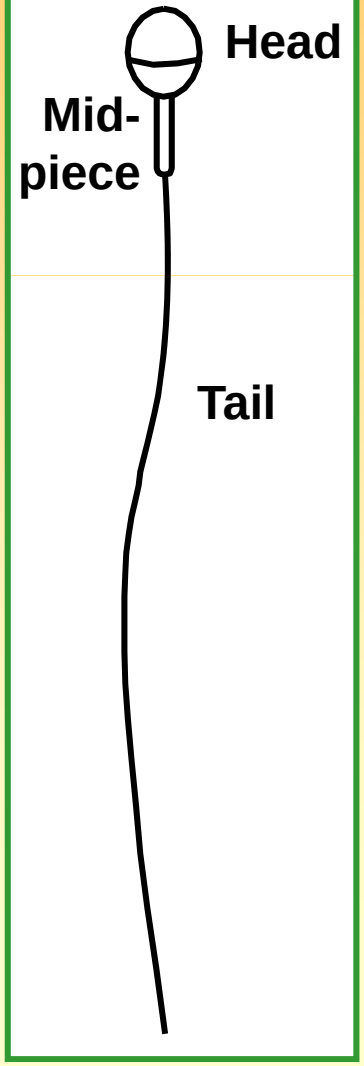
BACKGROUND

Sperm transport and seminal plasma

- **"Testicular sperm" need to undergo more maturation steps before they are ready to fertilize**
- **Transported from the testes to the epididymis, where they mature, and acquire the *ability* to swim**
- **Then moved to the vas deferens, for storage**
- **At ejaculation, the sperm are transported out of the vas and mix with accessory gland secretions:**
  - **prostatic fluid (pH slightly acidic to neutral; contains citric acid and zinc)**
  - **seminal vesicle fluid (pH strongly alkaline; contains fructose)**

BACKGROUND

What the spermatozoon looks like (2)



- **The human sperm cell is about 70 µm long**
- **The nucleus is in the head – contains the 23 chromosomes**
- **It is the head which binds to the egg at fertilization**
- **Midpiece: the energy for motility is generated**
- **Tail: motility – the beat is initiated just behind the midpiece, and then propagated along the tail**

BACKGROUND

Sperm function

- **The ejaculated sperm pass through the cervix, then the uterus, and enter the oviduct**
- **The fertilizing sperm swims through the layers of cells around the egg (cumulus and corona), and reaches the zona pellucida**
- **The sperm then loses the front membranes of its head (the "acrosome reaction"), binds to the zona, then forces its way through the zona to the egg membrane**
- **When the sperm head binds to the egg membrane, its tail stops beating, and the egg incorporates the whole sperm cell**
- **The egg unpacks the sperm, then the male and female pronuclei form.**

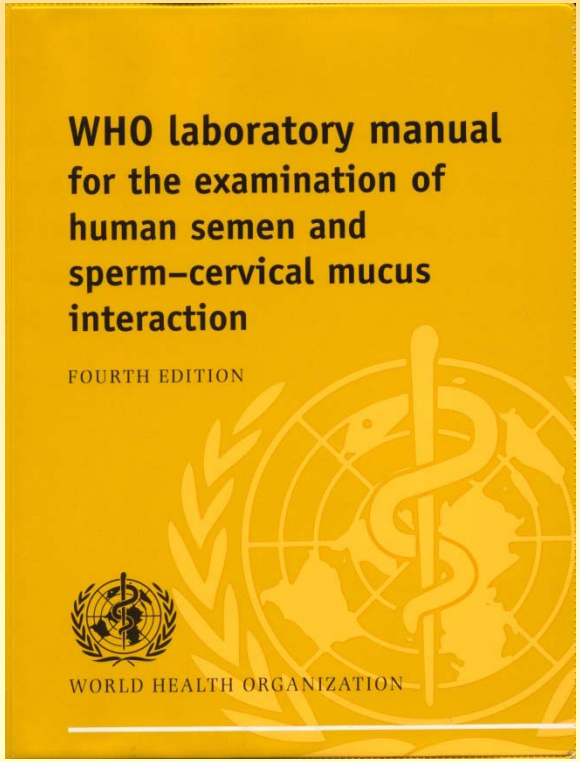
WHY PERFORM SEMEN ANALYSIS?

- Diagnosis of sterility
- Diagnosis of infertility
- Prognosis for fertility
- Identify treatment options:
  - surgical treatment
  - medical treatment
  - assisted conception treatment

Therefore = a screening test to help direct management.

What is the “standard” approach to semen evaluation?

International minimum standards are, by consensus, the World Health Organization’s Lab Manual (Fourth edition).



- **Focus is on standardization with expanded section on quality control.**
- **Methods amenable for use in any (“third world”) country.**
- **Basic infertility work-up.**

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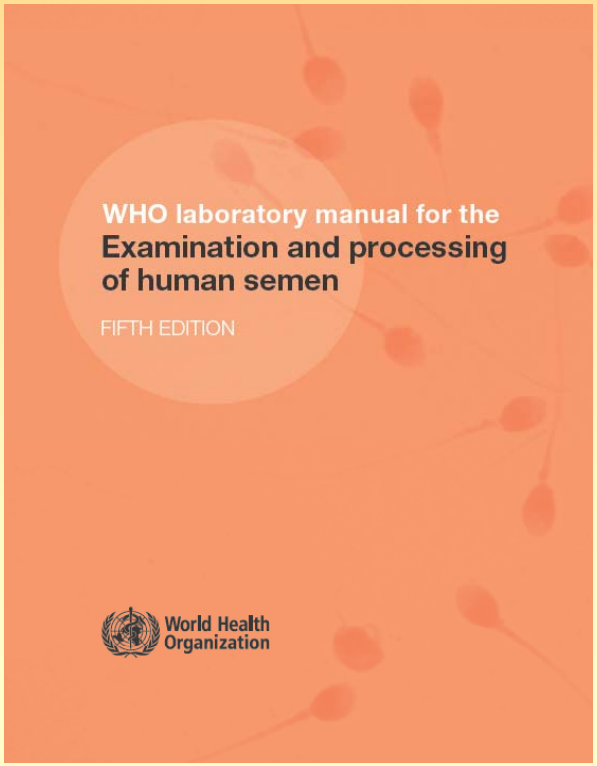


- **Focus is on standardization with expanded section on quality control.**
- **Methods amenable for use in any (“third world”) country.**
- **Basic infertility work-up.**

SEMEN ANALYSIS

Sample Collection

For a meaningful result, semen samples must always be collected under standardized conditions:

- **the container has to be sterile and known NOT to be spermotoxic (i.e. provided by the lab)**
- **the man must have had 3 – 5 days of abstinence**
- **the man must have washed his hands before collection (particularly if microbiological analysis is requested)**
- **the man must NOT have used lubricants.**
- **the sample must be kept at 37°C until analysis, which begins ideally within 30 min, but absolutely within 60 min, of ejaculation**

SEMEN ANALYSIS

Sample Handling

- **The semen sample should be mixed gently during the liquefaction period to promote liquefaction**
- **The sample should NEVER be vortexed (the only exception is for the fixed prep for concentration assessment)**
- **The sample should NEVER be "needled" – if it is too viscous to work with, a known volume of sperm buffer should be added and the sample mixed gently. The added volume must be included in the sperm concentration calculation**

SEMEN ANALYSIS

Macroscopic Evaluation

- **There are several macroscopic evaluations which give useful diagnostic information about the sample:**
  - **Appearance**
  - **Odour**
  - **Liquefaction**
  - **Volume**
  - **Viscosity**
  - **pH**

SEMEN ANALYSIS

Macroscopic Evaluation - Appearance

- **Swirl the sample, still in the collection jar, gently**
- **Hold the jar up to look through its bottom**
- **Note the following:**
  - **Colour (normal = white to grayish-yellow) – if there is blood present, it may range from pink to brown**
  - **Opacity / translucence (normal = tends to opaque)**
  - **Whether mucus streaks or cell clumps are present**

SEMEN ANALYSIS

Macroscopic Evaluation - Odour

- **Different people have different abilities to smell semen, so this cannot be standardized**
- **However, when the lid is taken off the collection jar, it should be noted if there is a strong smell of urine or of putrefaction**
- **Samples collected after a prolonged abstinence period (i.e. several weeks) are likely to have a stronger odour**

SEMEN ANALYSIS

Macroscopic Evaluation – Liquefaction & Viscosity

- ***Liquefaction*** **is the breakdown of the gel portion of the seminal plasma – the enzymes for this are in the prostatic fluid**
  - **A sample with incomplete liquefaction has a gelatinous material in a liquid base – this can be seen when the sample is swirled for the appearance assessment**
- ***Viscosity*** **is related to the fluid nature of the whole sample**
  - **This is rated subjectively according to the length of the thread of semen produced when the sample is allowed to run back out of the volumetric pipette used to measure the ejaculate volume**

SEMEN ANALYSIS

Macroscopic Evaluation - Volume

- **The volume of the sample should be measured to allow an accurate determination of the sperm number**
- **This is most easily assessed using a warmed disposable volumetric pipette (which is sterile and known NOT to be spermotoxic)**
- **After the sample is measured, allow it to run back into the collection jar, noting its viscosity (a normal sample will have some viscosity – i.e. not watery, but it will flow easily)**

SEMEN ANALYSIS

Macroscopic Evaluation - pH

- **pH is important because sperm die at pH < 6.9**
- **The pH of liquefied semen is normally determined using test strips (we use EM Science ColorpHast type, pH 6.5–10.0)**
- **We usually measure pH after volume and viscosity – by touching the "emptied" volumetric pipette to the test strip**
- **The normal pH range is 7.2–8.4**
- **Inflammatory disorders of the accessory glands can take the pH outside of this range**

SEMEN ANALYSIS

The wet preparation – set-up

- **Place 10µl of thoroughly mixed, liquefied semen on a microscope slide and cover with a 22x22mm No 1½ coverslip**
- **There are several important points to keep in mind:**
  - **The quality of sperm motility is affected by temperature – the lower the temperature, the poorer the motility, and then cold shock starts to occur at around 15°C. So great care must be taken to ensure that the slides and coverslips, as well as the pipette tips are kept at 37°C**
  - **The assessment must start as soon as the flow stops – if this is >1 minute, then a new wet prep must be made**
  - **Microscope: phase contrast optics and a heated stage**

SEMEN ANALYSIS

The wet preparation – assessments

- **The characteristics assessed are:**
  - **Motility (to be discussed later)**
  - **Sperm aggregation (random clumping) – "some" is normal, but large clumps (each with hundreds of sperm) is abnormal**
  - **Spermagglutination (between specific sites) – could suggest the presence of antisperm antibodies.**
  - **Round cells: should be <1 per 40× field (~ 1 million/ml). If more abundant, a leukocyte test should be run**
  - **Epithelial cells: usually present in small numbers**
  - **Erythrocytes: should not be present**
  - **Debris: particles smaller than sperm head, may be plentiful**
  - **Bacteria and protozoa: presence indicates infection**

SEMEN ANALYSIS

Motility assessment - Background

- **This is the first assessment made on the wet prep**
- **If >10–15% of the sperm are clumped, just assess the free-swimming sperm (and note this on the report form)**
- **A repeat assessment should be made on a second wet prep**
- **Don't estimate, *count* (≥ 4 fields and 200 sperm per prep)**
- **Use a 40× objective, and phase contrast optics**
- **Make a *random selection* of the fields that are assessed**
- **Assess fields that are away from the coverslip edge**
- **Count only those sperm that were in the field at one moment in time (you have to be quick!)**
- **There must be agreement to within 10% between duplicates**

SEMEN ANALYSIS

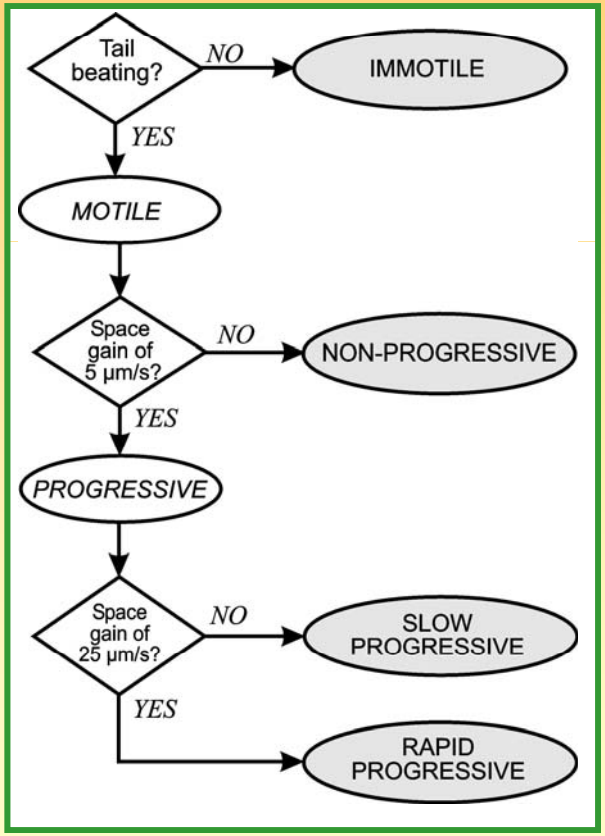
Motility assessment - types

- **% motile = the proportion of sperm with tail movement**
- **Progression rating = the grade of progression shown by the majority of the sperm: this can be from 0 (all immotile) to 4 (all with rapid progression); or from *a* (rapid progression) to *d* (all immotile)**
- **Differential motility count = proportion of sperm in each of 4 motility classes (rapid progressive; slow progressive; non-progressive; immotile)**

SEMEN ANALYSIS

Motility assessment – differential motility

- **Differential motility classification is based on the distance swum over time:**
  - **Rapid progressive: > 25 µm/s**
  - **Slow progressive: 5 – 25 µm/s**
  - **Non-progressive: < 5 µm/s**
  - **Immotile: no flagellar movement**
- **The sperm head is about 5 µm long, so the rapid sperm have a net gain of 5 head lengths / second**



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Motility assessment – monitor

- **Alternatively, use a video monitor with a grid for the differential motility assessments – so we do them off the screen.**
- **Each square is the equivalent of 25 × 25 µm (a stage micro-meter is used to set this up)**
- **This approach is very useful for training and for QC/QA**

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Motility assessment – lab sources of error

- **Have the definitions been implemented correctly?**
- **Are staff trained to classify progression?**
- **Internal quality control?**
- **How is velocity/progression assessed?**
- **Effect of temperature : *% motile = minimal***
  % progressive = slight
  % rapid = very large
- **Representative sample aliquots?**
- **Adequate number of sperm counted?**
- **Repeatability of duplicate counts?**
- **Calculations performed correctly?**
- **Precision of results?**
- **External quality assurance / proficiency testing?**
- **Uncertainty of results?**

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Motility assessment – interpretation

- **The WHO's Reference values for motility are:**
  - **50% or more with progressive motility; or**
  - **25% or more with rapid progressive motility**
- **Assuming that all of the collection and laboratory factors have been controlled, a poor motility result may have negative implications for fertility.**
- **However, this should be confirmed by a repeat semen analysis, and the result should be interpreted with the rest of the semen analysis results**

SEMEN ANALYSIS

Sperm concentration- background

- **Sperm count = total number of sperm in the ejaculate**
- **Sperm concentration = number of sperm per ml**
  (not "density", which is an expression of mass / unit volume)
- **Most accurate method is volumetric dilution and haemocytometry**
- **Must use a positive displacement pipettor**
- **We make a 1 + 19 dilution with a fixative solution (allows the assessments to be batched - usually counted within two days)**

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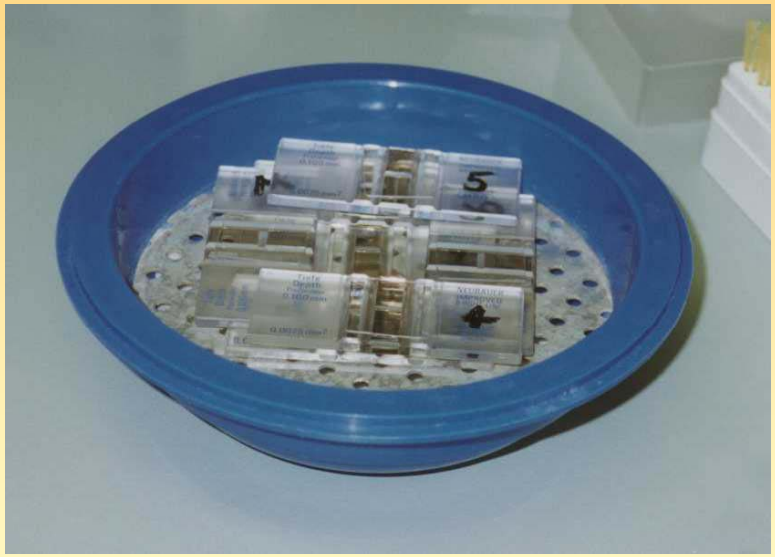
Sperm concentration - method

- **Fix the haemocytometer coverslip over the chambers – ensure interference fringes can be seen**
- **Vortex the fixed sample, load ~10 µl in both chambers**
- **Leave in a humid chamber for 10-15 minutes**
- **Count using a 20× objective and phase contrast optics**

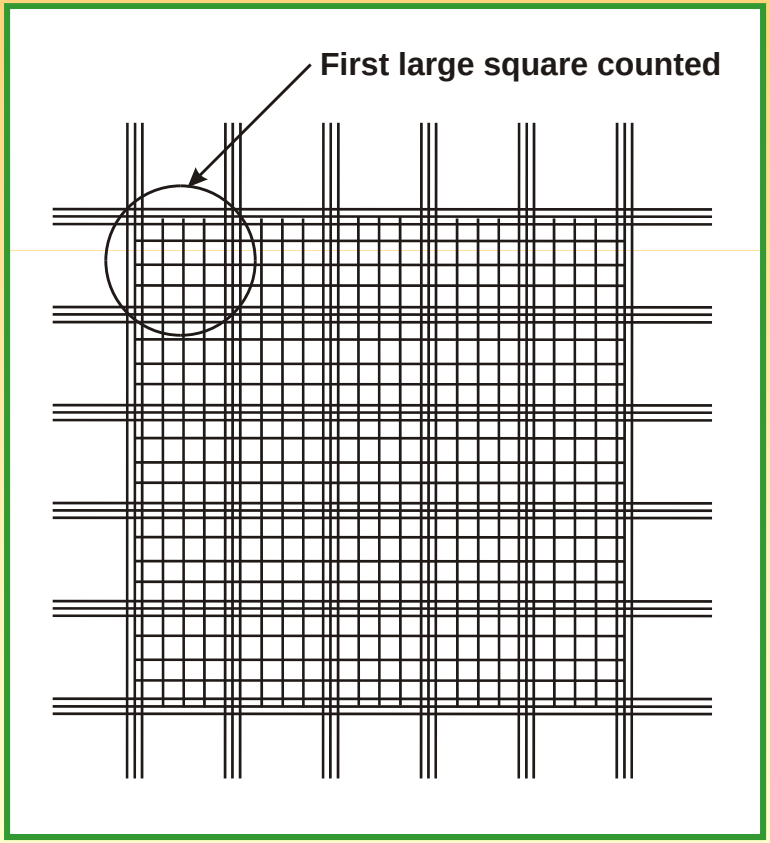


SEMEN ANALYSIS

Sperm concentration - haemocytometer

The number of squares assessed depends on the number of sperm counted in the first large square:

- **If < 10 counted, the whole grid is assessed**
- **If 10-40 counted, 10 squares are assessed**
- **If > 40 counted, 5 squares are assessed**



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Sperm concentration - calculations

- **If the counts of the two chambers are *not* within 5% of their average (i.e. the difference > 1/20 of their sum): discard, remix the sample, and set it up again**
- **If the two counts are in agreement, then the sum of the two counts is divided by the correction factor:**
  - **If 2 × 25 squares counted, divide their sum by 10**
  - **If 2 × 10 squares counter, divide their sum by 4**
  - **If 2 × 5 squares counted, divide their sum by 2**
- **This gives the sperm concentration in millions per ml**
- **Sperm count = concentration × total volume**

SEMEN ANALYSIS

Sperm concentration – lab sources of error

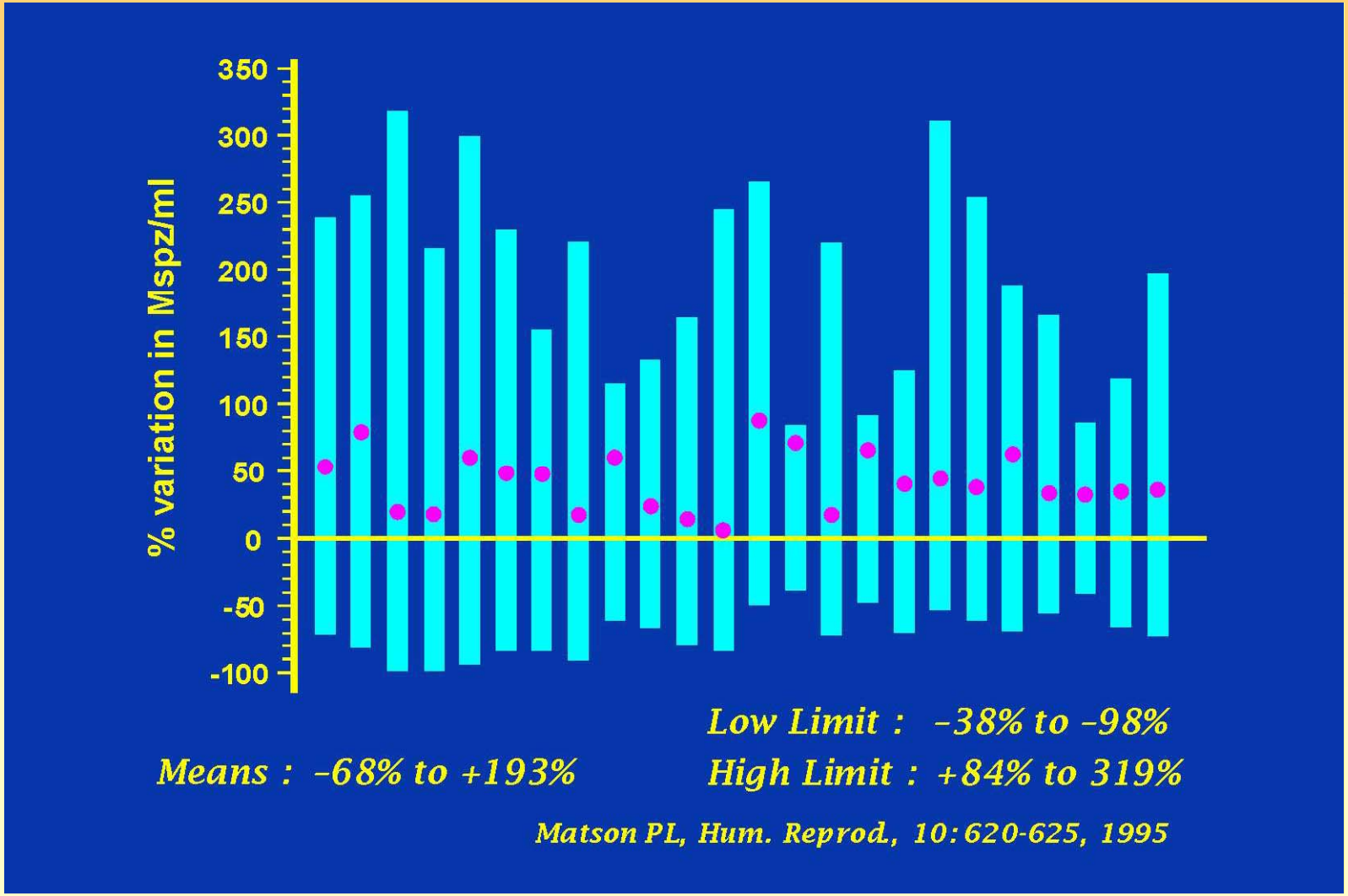
- **Sample aliquot representative of ejaculate?**
  - ***– semen homogeneous (mixed)?***
  - ***– accurate sample aliquot (N.B. viscosity)?***
  - ***– duplicate aliquot?***
- **Accurate dilution :**
  - ***– volumes of sample aliquot and diluent?***
  - ***– storage (airtight) / sperm bind to vial?***
- **Secondary sampling :**
  - ***– mixing of diluted aliquot?***
  - ***– duplicate aliquots?***
- **Preparation of counting chambers :**
  - ***– good chamber design/manufacture?***
  - ***– chamber loaded correctly &/or cover glass placed correctly?***
  - ***– adequate minimum number of cells?***
  - ***– repeatability of duplicate counts?***
- **Calculations correct?**
- **Precision of results?**
- **Uncertainty of measurement known?**

SEMEN ANALYSIS

Sperm concentration – interpretation

- **The WHO Reference values for:**
  - **Sperm concentration is $\geq 15 \times 10^6$ sperm/ml**
  - **Sperm count is $\geq 39 \times 10^6$ sperm per ejaculate**
- **A persistently low sperm concentration is associated with impaired fertility**
- **If a man has a sperm concentration $< 5 \times 10^6$ sperm/ml, the WHO recommends assessment for numerical and structural abnormalities of sex chromosomes**
- **Azoospermia can indicate a failure of spermatogenesis or blockage(s) in the male tract**

Variation in Sperm Counting



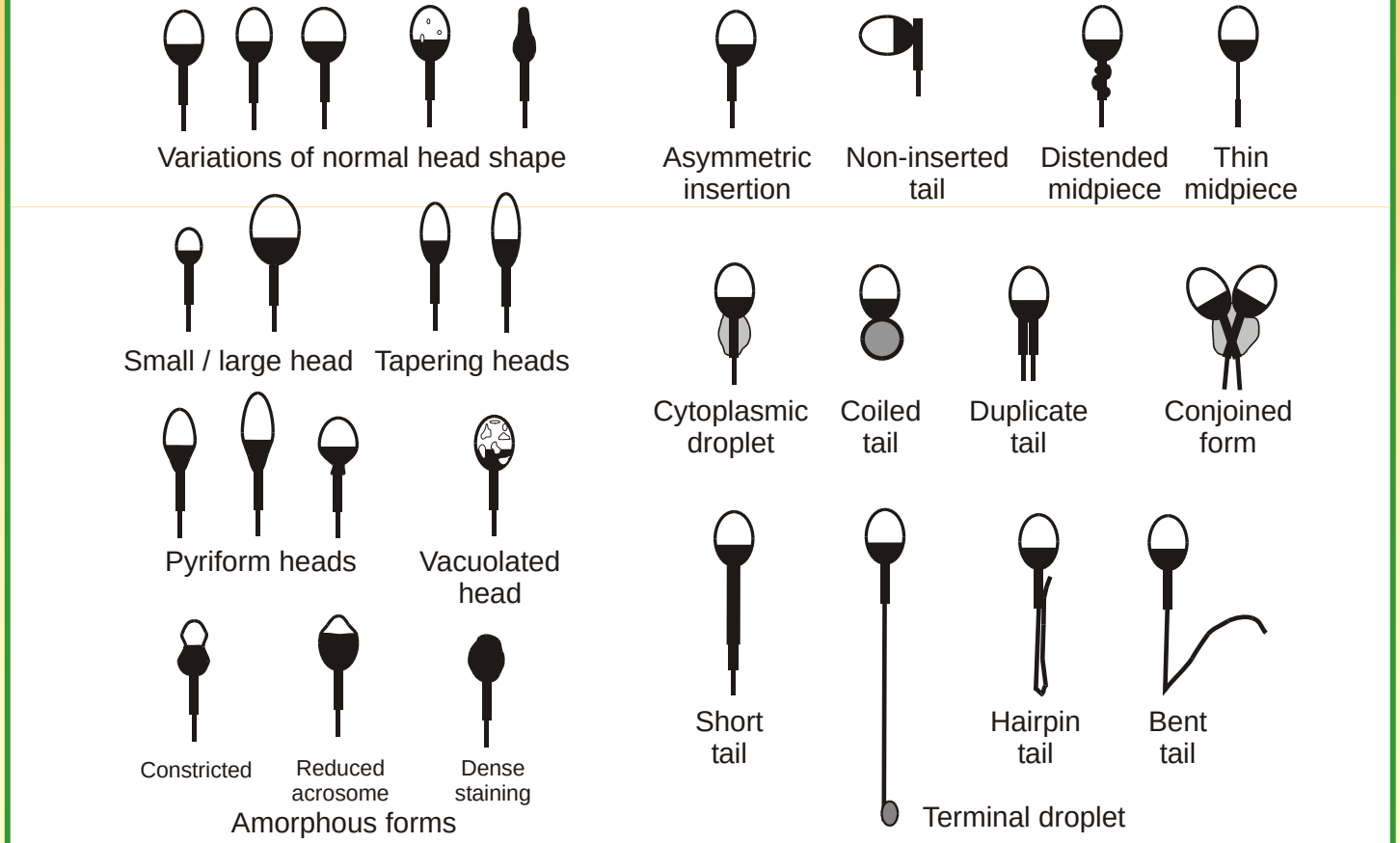
SEMEN ANALYSIS

Sperm morphology – background

- **Morphology is even more important than motility and concentration**
- **Because of the small size of the human sperm head, *must* use an air-dried smear which has been stained**
- **The Papanicolaou method is best**
- **Prepared samples are assessed using a 100× oil-immersion objective under bright field optics**
- **The WHO recommends that 200 spermatozoa are counted per sample (and says that 2 × 200 is better)**
- **Fields for counting must be selected at random**
- **When counting, remember about the normal distribution**

SEMEN ANALYSIS

Sperm morphology – morphology types



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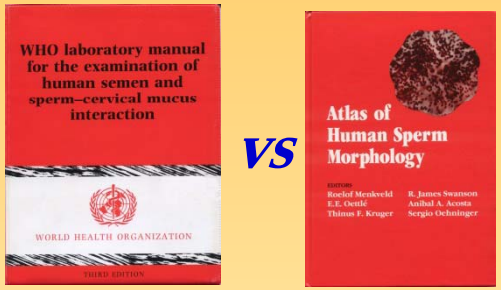
Sperm morphology – assessment

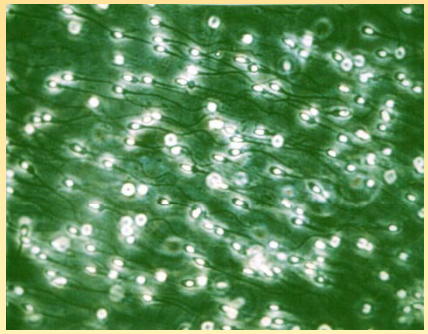
- **Even "good" samples will have <20% normal forms**
- **But we know that these sperm from these samples can fertilize eggs – so is the morphology any help at all?**
- **Yes it is – but there are a few things to think about:**
  - **The result will indicate a probability, not an absolute**
  - **You have to count 200 sperm to discriminate reliably between, say, 3% and 5% ("Strict Criteria" cut-off 4%)**
  - **The dynamic range of 4 – 15% is not very large, and means that it is difficult to make a meaningful assessment**

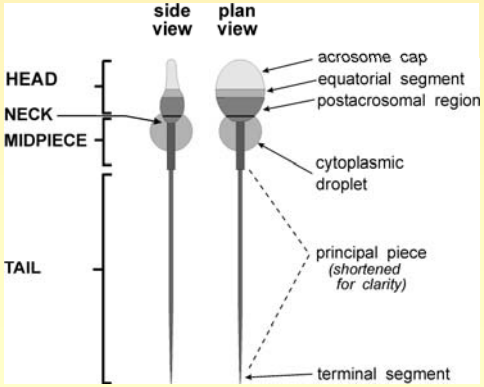
Diff quick staining kit

SEMEN ANALYSIS

Sperm morphology – assessment schemes







- **Tygerberg scheme defines normal sperm and counts the number that meet these criteria.**
- **WHO'92 looks for, and counts, defects that might affect sperm function in each region of a sperm – normal sperm are those that have no recognizable defects.**
- **Expert direct comparison study between the schemes* revealed little difference.**
- **Issues with counting error and uncertainty of measurement, especially if only 100 sperm counted.**
- **Need additional information, e.g. Acrosome Index or Teratozoospermia Index (TZI) when dynamic range of % normal forms is very limited.**

*** Mortimer & Menkveld (2001) J. Androl. 22:192-205.**

SEMEN ANALYSIS

Sperm morphology – TZI

- **The Teratozoospermic Index is an expression of the average number of abnormalities per abnormal sperm**
- **Each sperm cell is assessed for an abnormality in the head, neck/midpiece, or tail, and for a cytoplasmic droplet**
- **If it does not have any of these abnormalities, it is “normal”**
- **If it does have an abnormality, it is “abnormal”, and we score each abnormality. So, if a cell has an abnormal head and tail, it is counted as 1 cell, and 2 abnormalities**
- **Then, (total # abnormalities) / (total # sperm) = TZI**
- **A TZI > %80 has been associated with poor sperm fertilizing ability *in vivo* and *in vitro***

SEMEN ANALYSIS

Semen biochemistry

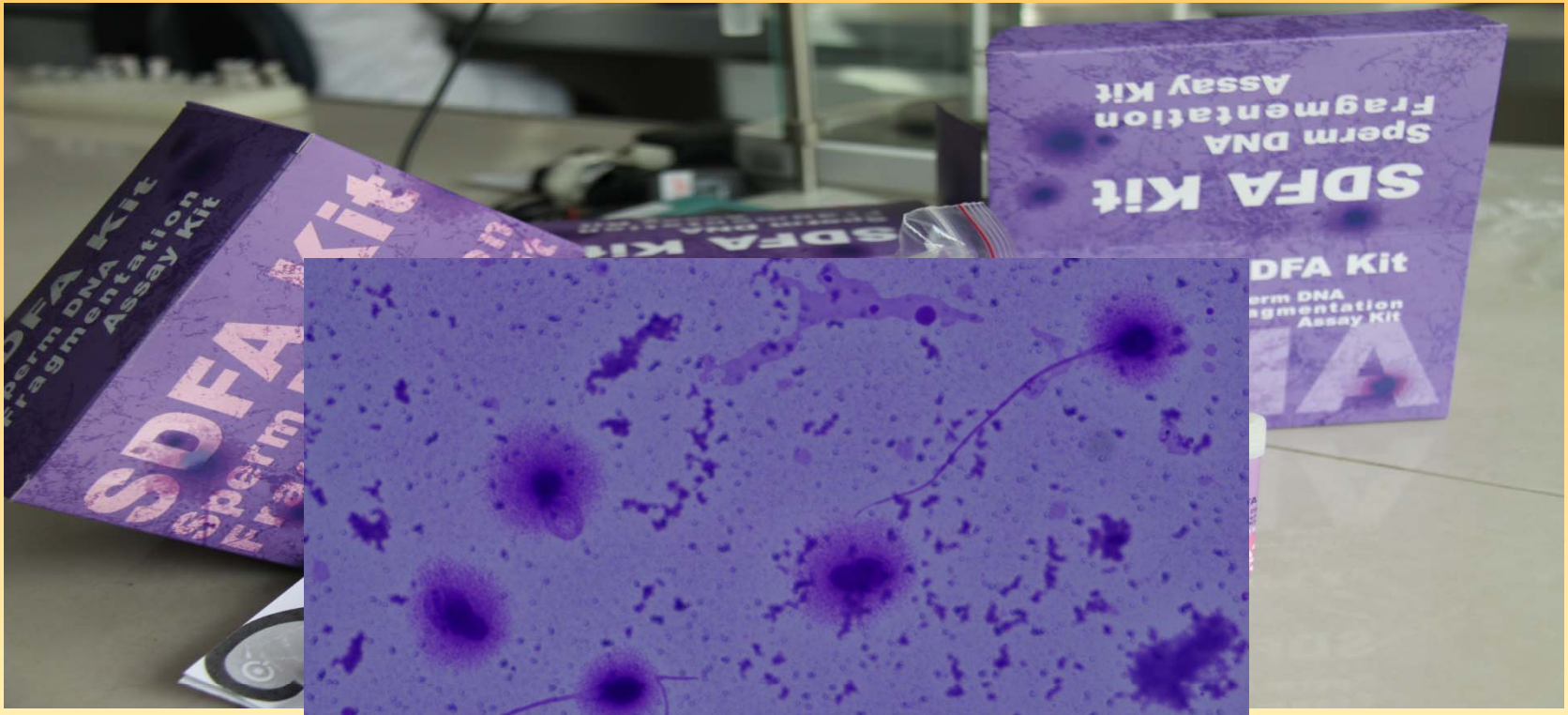
- **Acid phosphatase: marker for prostatic function**
- **Citric acid: can indicate prostatic function – low levels may indicate dysfunction or a prostatic duct obstruction**
- **Zinc: marker for prostatic function – *colorimetric assay (WHO)***
- **Fructose: marker for seminal vesicle function, and is a substrate for sperm metabolism – *spectrophotometric assay (WHO)***
- **α-Glucosidase: secreted exclusively by the epididymis and so is a marker for epididymal function – *spectrophotometric assay (WHO)***

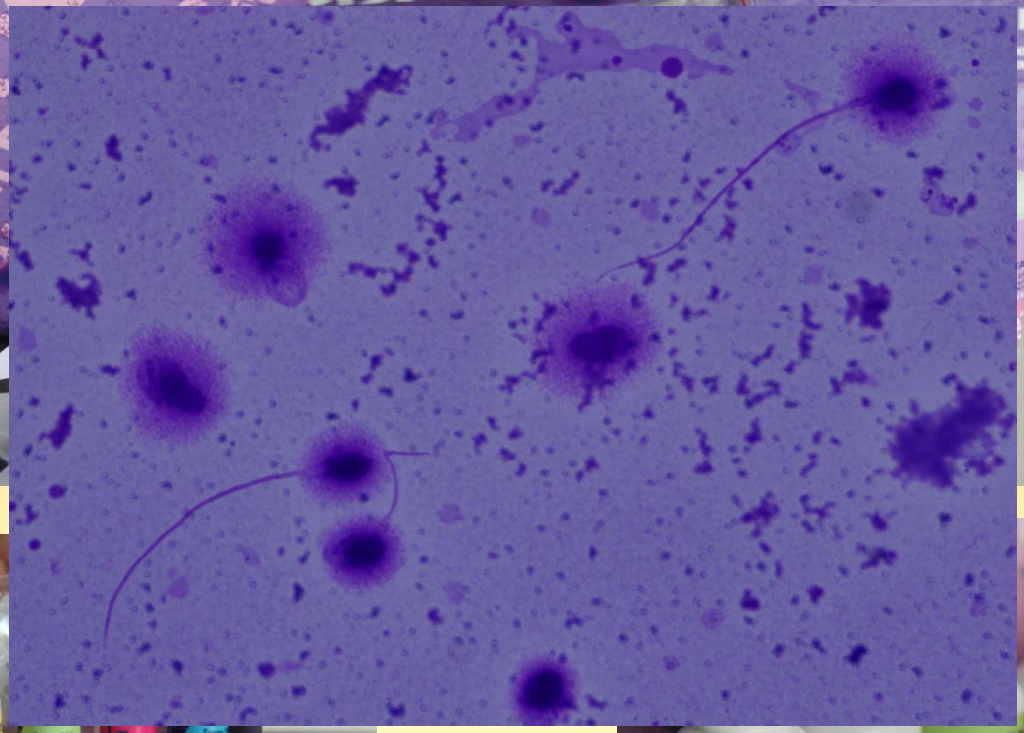
SEMEN ANALYSIS

Sperm Vitality

- **Used in samples with low total motility (<50%)**
- **Distinguishes between samples with live, but immotile sperm (e.g Kartagener syndrome), and those with lots of dead sperm (could result from: sperm senescence; exposure to detergents or lubricants; or spermotoxic antibodies)**
- **Also provides a check on the accuracy of motility assessments: % live should be slightly higher than % motile**
- **Usually performed using a vital stain, such as eosin Y, with a counterstain (nigrosin) – although could use a fluorescent DNA stain, such as H33258.**

SDFA Kit
Sperm DNA Fragmentation Assay Kit


SDFA Kit
Sperm DNA
Fragmentation
Assay Kit



SDFA Kit
Sperm DNA
Fragmentation
Assay Kit

SDFA
Solution A
Solution B
Solution C
Solution D
Solution E

SEMEN ANALYSIS

Retrograde ejaculation

- **In some men, the semen passes back into the bladder at ejaculation - this is confirmed by examination of a sample of post-ejaculatory urine**
- **The man must take sodium bicarbonate the day before, and the day of, his appointment – to alkalinize his urine**
- **Before collection, he should pass urine and then wait until he feels there is some urine in his bladder before masturbating**
- **He then collects a urine sample for analysis**
- **Assess volume and pH of the urine**
- **Centrifuge (600*g* for 10 min), resuspend pellets to a total of 20ml with sperm buffer + protein**
- **Re-spin, resuspend pellets and combine – add buffer to a final volume of 1ml**
- **Perform a standard semen analysis with this suspension**

SEMEN ANALYSIS

Post-vasectomy analysis

To determine the sperm concentration when few/none expected:

- **centrifuge as much of the sample as possible (1000 *g* for 15 min) in a disposable conical centrifuge tube – note the exact volume used**
- **Remove most of the supernatant - note volume removed**
- **Make at least 5 wet preps (10µl of resuspended pellet under a 22 × 22mm coverslip)**
- **Search through at least 5 wet preps**
- **If anything that looks like a sperm is seen – the report is "not clear"**
- **Final decision is the responsibility of the embryologist**

SEMEN ANALYSIS

What tests should always be done?

Always:

- **Semen volume**
- **Sperm concentration**
- **Differential motility**
- **Morphology**

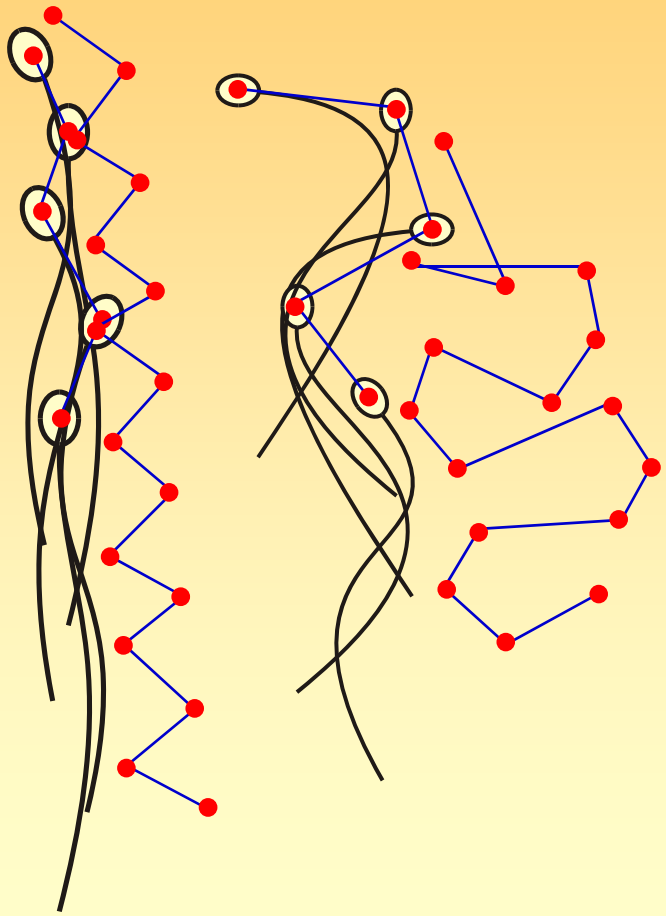
If there is an indication:

- **White cells**
- **Vitality**

RESEARCH & NEW TECHNIQUES

Sperm kinematics

- **The way sperm swim affects their fertility**
- **To get through the cervix, they have to swim in a straight path**
- **To get through the outer layer of the egg, they have to generate a lot of power – this is seen as hyperactivated motility**



RESEARCH & NEW TECHNIQUES

Sperm kinematics

- **The sperm tracks are analyzed and a number of kinematic parameters are derived, including:**
  - **Velocity (VCL, VSL and VAP)**
  - **Velocity ratios (expression of the path shape and regularity)**
  - **Amplitude of lateral head displacement**
  - **Beat/cross frequency**
- **The proportion of sperm in a sample which meet particular kinematic criteria is used to predict (failure) of:**
  - **Mucus-penetrating ability**
  - **Hyperactivation (a marker of sperm function)**

RESEARCH & NEW TECHNIQUES
CASA

- **Computer-aided sperm analysis**
- **Able to assess the kinematics of hundreds of sperm in a couple of minutes**
- **Means that these tests can be part of the infertility work-up**

STANDARDIZATION & QUALITY CONTROL

Fundamental requirements of all laboratory tests :

- **appropriate and robust methodology**
- **careful training**
- **operator experience**

} ***accuracy & precision***

- **suitable assay control limits**
- **regular internal comparisons** = ***IQC***
- **regular external comparisons** = ***EQA***
- **cost effectiveness**

College of Reproductive Biology Inaugural Meeting, Houston, May 1997

CONCLUSION

- **Semen analysis is an important laboratory test and should be thought of in the same way as any other diagnostic assay**
- **It is used in determining treatment plans for infertility**
- **The results can therefore have a huge impact on the level of intervention, with the associated emotional and financial costs to the couple**

Samples / Motility No. / Grade	245	246	247	248
a	25.3	37.1	24.3	66.7
b	21.2	25.1	34.3	9.2
c	11.1	13.7	14.2	4.6
d	42.2	23.4	26.5	25.6